AWARD NUMBER: W81XWH-16-1-0372

TITLE: Early Detection of Prostate Cancer with New Nanoparticle-Based Ultrasound Contrast Agents Targeted to PSMA

PRINCIPAL INVESTIGATOR: James Basilion PhD

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, OH 44106

REPORT DATE: August 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2017	Annual	1 Aug 2016 - 31 Jul 2017

4. TITLE AND SUBTITLE
Early Detection of Prostate Cancer with New Nanoparticle-Based Ultrasound Contrast Agents Targeted to PSMA

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0372

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
James Basilion PhD

james.basilion@case.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
.
Case Western Reserve University
Cleveland, OH 44106

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The development of a new tool to accurately delineate cancer within the prostate is urgently needed to shape the future of PCa workup and biopsy guidance. To provide a more effective, practical tool for clear identification of PCa for biopsy, this IDEA proposal will develop a nano-sized US contrast agent (called a nanobubble – NB) targeted to the prostate specific membrane antigen (PSMA) via a new highly selective ligand. The targeted NBs are similar in structure to clinically used microbubbles (MB) and are clearly visible on clinical US at comparable frequencies of 3-12 MHz. However, in stark contrast to MB which remain in the vasculature, the <200 nm NB size enables them to extravasate into the tumor parenchyma and directly bind to cancer cells. This can result in higher accumulation of contrast at the tumor itself leading to better resolution and detection of PCa. The PSMA-targeted NB has the capacity to revolutionize PCa imaging, since US is so broadly available, low cost, and safe. Importantly, US is already frequently utilized in PCa biopsy procedures, and the same exact equipment and process can be applied for NB imaging, thus lowering cost and expediting development and clinical translation.

15. SUBJECT TERMS-
None listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	32	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The development of a new tool to accurately delineate cancer within the prostate is urgently needed to shape the future of PCa workup and biopsy guidance. To provide a more effective, practical tool for clear identification of PCa for biopsy, this IDEA proposal will develop a nano-sized US contrast agent (called a nanobubble – NB) targeted to the prostate specific membrane antigen (PSMA) via a new highly selective ligand. The targeted NBs are similar in structure to clinically used microbubbles (MB) and are clearly visible on clinical US at comparable frequencies of 3-12 MHz. However, in stark contrast to MB which remain in the vasculature, the <200 nm NB size enables them to extravasate into the tumor parenchyma and directly bind to cancer cells. This can result in higher accumulation of contrast at the tumor itself leading to better resolution and detection of PCa. The PSMA-targeted NB has the capacity to revolutionize PCa imaging, since US is so broadly available, low cost, and safe. Importantly, US is already frequently utilized in PCa biopsy procedures, and the same exact equipment and process can be applied for NB imaging, thus lowering cost and expediting development and clinical translation.
The specific objective of this project is to optimize PSMA-targeted nanobubbles for US detection of PCa. Specifically we will: 1) formulate targeted NBs and characterize NB parameters in human PCa lines (e.g. bubble echogenicity and stability, specificity and longevity of cell targeting); and 2) compare the US detection properties of PSMA-targeted NB to untargeted NBs and clinical MB agents (Definity®) in both flank and orthotopic *in vivo* PCa models. This proposal represents the first step in establishing these NB agents as tools for guided biopsies of the future, and will confirm that the NBs can indeed delineate a target lesion of concern more effectively on US than current standard of care. In the long term, biopsies improved by the PSMA-targeted US contrast agents could lead to increased detection of high grade tumors and tumor staging, and lower morbidity.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Ultrasound contrast agent, nanobubbles, microbubbles, PSMA, prostate cancer, imaging, molecular imaging, early detection, biopsy guidance.

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Three specific aims support the project objectives:

Aim 1: Formulate and characterize PSMA targeted NBs (PSMA-1-NB): We will determine the optimal NB formulation parameters once they are functionalized by the PSMA-1 ligand. We seek a stable

formulation that will be successfully targeted to PSMA with high yield. The formulation needs to be visible on US and sustain its signal for at least 1 hr and be 200 nm.

Aim 2: Establishment of NB in vivo performance: The primary objective of this aim will be to characterize and maximize PSMA-1-NB signal at the PSMA positive tumors and minimize in PSMA negative ones. We will compare these to MB performance in the same tumors.

Aim 3: Application of targeted NBs in detection of orthotopic PCa: Because a clinically relevant tumor microenvironment and tumor heterogeneity as well as the delineation of the tumor within the prostate tissue play crucial roles in the outcomes of the proposed imaging technique, it is necessary to carry out an imaging study on an orthotopic model of PCa. This model has been established by the Basilion lab in mice, and we will build upon their extensive knowledge of this model to test the imaging strategy. We will acquire US images and will then utilize 3D whole mouse cryoimaging [52] to determine the efficacy of segmentation of the tumor and prostate tissue.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Proposed Statement of Work (Months 1-12)

In the application, we proposed to focus primarily on formulation and optimization of the nanobubble contrast agents during the first 12 months. During this time, we have accomplished nearly all of the sub-tasks proposed. These are labeled with a green dot (●). The sub-tasks which have a yellow dot (●) are currently underway. We describe the details of our major accomplishments below.

<u>Task 1</u>: Formulate and characterize stable NBs targeted to PSMA receptor (months 1-10)

1a. Develop optimized combination of lipids and surfactants to form NBs (months 1-2) ●
1b. Optimize conditions for fluorescent labeling of NBs (months 1-2) ●
1c. Determine best strategy for targeting NBs to prostate cancer with the PSMA antibody (months 2-3) ●
1d. Formulate stable targeted NBs (months 2-3) ●
1e. Characterize NB properties (months 1-3) ●
1f. Measure NB stability (months 1-3) ●
1g. Refine techniques as needed to achieve optimized NB <100 nm and stable for >1hrs (months 1-4) ●
1h. Carry out acoustic characterization of NBs (months 4-5) ●
1i. Initiate culture of PCa cell lines in collaboration with J Basilion (months 4-7) ●
1j. Confirm PSMA expression on cells (months 4-5) ●
1k. Determine targeted NB attachment on cultured cells under <u>static</u> conditions (months 4-7) ●
1l. Determine targeted NB attachment on cultured cells under <u>flow</u> conditions (months 7-10) ●

1m. Measure NB adhesion using flow cytometry (months 5-9)
1n. Statistical analysis of results and refinement as needed to achieve desired criteria (months 9-10)

<u>Task 2:</u> Evaluate contrast agent properties *in vivo* in flank prostate cancer model in mice (months 11-24)

2a. Prepare and attain IACUC approval for mouse xenograft studies (months 9-11)
2b. Inject tumor cells in flank in male mice in collaboration with J Basilion (months 11-15)
- 5 groups (PCa cell lines, 3 types of bubbles) of n=9 for each endpoint. Total of 126 mice

2c. Carry out biodistribution studies with FMT, ultrasound imaging and ex vivo tissue analysis (months 10-14)
2d. Measure bubble dynamic parameters (months 10-14)

1. Overview of research accomplished pertaining to Task 1

During the past year, we have carried out a substantial amount of work in this area. Our initial efforts focused on optimization of the nanobubble stabilizing shell, which is a critical component which influences the nanobubble longevity and interaction with ultrasound. To carry out these studies, we first examined the effect of two different lipids (DPPC and DBPC) as well as the combination of lipids and lipid to Pluronic ratio on the stability and size distribution of the nanobubbles. We also formulated hybrid lipid and polymer (polyacrylamide)-stabilized nanobubbles and examined their morphology under cryo electron microscopy (cryo-EM), and we examined bubble morphology following exposure to ultrasound, to confirm their size and presence of gas inside of the lipid core. Finally, we studied two different ways of isolating the nanobubble population and removing any bubbles which were larger than 1 micron. Here we refined the centrifugation protocol and we also utilized a set of filters to remove the large bubble population and reduce polydispersity.

Pertaining to synthesis and conjugation of PSMA-1 ligand to the phospholipids, we first confirmed PSMA-1-cys ligand synthesis via MALDI-TOF analysis. We concurrently confirmed conjugation of thiol groups to the commercially obtained DSPE-PEG-maleimide lipids using a standard Ellman's assay, which is used to quantify the number or concentration of thiol groups in a sample. We then confirmed the conjugation of the DSPE-PEG—MAL lipid to the PSMA-1 ligand, also using MALDI-TOF analysis. In addition, the cell lines which we will utilize for *in vitro* as well as *in vivo* work were cultured, and the expression of PSMA was validated using western blotting.

Following the initial fundamental experiments to stabilize nanobubbles and develop and validate the targeting ligand, optimized nanobubbles were decorated with the PSMA-1 ligand. The nanobubble echogenicity, acoustic stability and size were characterized. Optimization of ligand density was carried out in live cells using an ELISA-like assay and the output was analyzed using a fluorescence plate reader. Confirmation of binding in cells was confirmed using fluorescence microscopy as well as a competition binding assay between free PSMA-cys and PSMA-1-conjugated nanobubbles (PSMA-1-NB).

1.1. Lipid acyl chain length improves stability of nano-sized ultrasound contrast agents *in vitro*

Ultrasound contrast agents require additional optimization with respect to their echogenicity, stability and size to make them suitable for cancer molecular imaging in extravascular applications. We had previously developed lipid and surfactant-stabilized perfluorocarbon gas nanobubbles (NB) capable of extravasating the permeable vasculature of tumors in contrast to conventional microbubbles which are too large (1-10 μm) to enter the interstitial space.[1,2] The objective of this set of experiments was to increase the stability of the NBs by optimization of the lipid hydrophobic chain length to improve the bubble shell in-plane rigidity. Specifically, based on previous microbubble literature, we replaced 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), a 16 carbon chain lipid, with 1,2-dibehenoyl-*sn*-glycero-3-phosphocholine (DBPC), a 22 carbon chain lipid.[3]

Methods**:** Nanobubbles were made by agitation of lipids, Pluronic L10, and glycerol in the presence of C_3F_8 gas. The mass ratio of lipids in the DPPC containing bubbles was 4:1.5:1:1 mg for DPPC, DPPE, DPPA and PEG respectively per vial of bubbles. The mass ratio of lipids in the DBPC containing bubbles was 6.1:2:1:1 mg per vial of bubbles, where DPPC is completed replaced by DBPC. The bubbles were imaged in an acrylamide phantom (**Fig. 1.A**) in PBS via contrast harmonic imaging (Toshiba, 12 MHz, MI 0.1).

Results and Discussion: Increasing the acyl chain length of the most prominent lipid in our formulation significantly improved (p>0.001) the half-life of our nanobubbles from 1.4 minutes to 5.8 minutes (**Fig 1.B-C**). This supports what previous literature has found to be true of microbubbles and shows that the same trends are found in nano-sized bubbles. We hypothesize that the longer chains increase lateral cohesion forces, which increases the bending modulus and decreasing lateral density fluctuations of the bubble.[3] The decreased bending modulus lessens buckling of the lipid monolayer, which prevents dissolution. Additional in depth experiments are required to support this hypothesis.

Conclusions: Increasing length of the acyl chain of the most prominent lipid from 16 to 22 carbons, while maintaining about the same molar ratios of all the lipids in the formulation resulted in a 4-fold improvement in half-life of nanobubbles under near continuous US exposure. Future studies will investigate the effects of acyl chain length on bubble stability and accumulation in cancerous tumors *in vivo*.

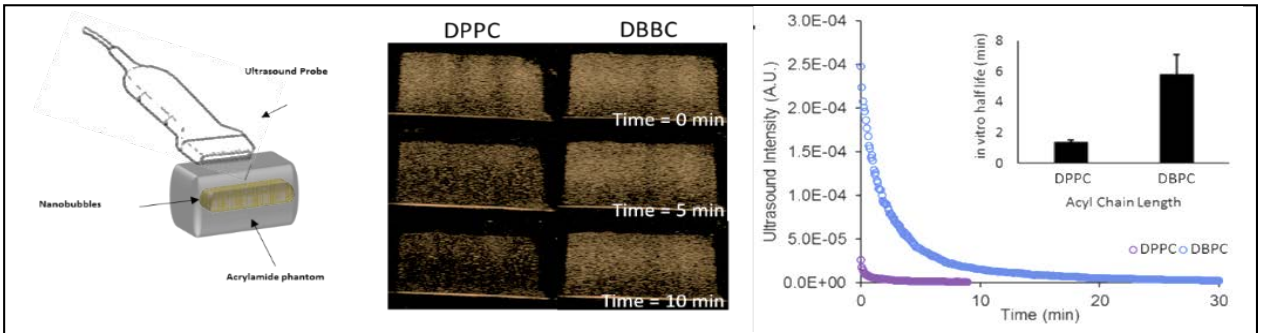


Figure 1. Effect of lipid acyl chain length on nanobubble stability under ultrasound exposure. (A) Schematic of acrylamide phantom used to measure in vitro stability. (B) Typical ultrasound images of nanobubbles at different time points. (C) Representative ultrasound time intensity curves and average half-life of each formulation.

1.2. Effect of the Surfactant Pluronic on the Stability of Lipid-Stabilized Perfluorocarbon Nanobubbles Due to their 1-10 μm size range, microbubbles (MBs) have limited use in cancer detection and treatment. To expand contrast enhanced US capabilities, we have developed sub-

micron contrast agents via the addition of Pluronic, a nonionic triblock copolymer surfactant, to the phospholipid shell stabilizing perfluoropropane (C_3F_8) gas (**Fig 2A**). NBs, with diameter of ~200nm, can take advantage of the EPR effect, extravasate the leaky tumor vasculature and accumulate in tumors. Prior work has shown that bubble echogenicity and stability are, in part, dependent on the surface tension of the stabilizing shell. In this study, we evaluate the effect of Pluronic on surface tension of the lipid films and how its presence in the NB shell affects echogenicity and signal decay at clinically-relevant imaging frequencies.

Methods: Pluronic L10 (MW 3200, PPO/PEO units of 49.7/7.3), at three Pluronic:lipid molar ratios (0.02, 0.2, and 0.4), was incorporated into the lipid film composed of a mixture of DPPC, DPPE, DPPA and DSPE-PEG. Bubble diameter was measured with dynamic light scattering (DLS). The surface tension of each composition was measured using pendant drop tensiometry. To test the effect of Pluronic concentration on bubble stability, NBs with the same Pluronic:lipid ratios were formulated by hydrating the lipid mixture described above with the appropriate Pluronic concentration and exchanging air with C_3F_8. Bubbles were then activated using mechanical agitation and imaged in PBS inside an agarose phantom using a standard diagnostic US scanner (Toshiba Aplio) in contrast harmonic mode at 12 MHz, MI 0.1, and 0.2 frames per second.

Results, Discussion and Conclusions: The incorporation of Pluronic L10 significantly decreased the surface tension, especially at a ratio of 0.2, where this value decreased by 27% ($p < 0.0001$) (**Fig 2 B**). This led to a significant decrease in the signal decay over time resulting in a stability increase of 39% ($p < 0.0001$) (**Fig 2C-D**). The Pluronic had little impact on size; NBs had an average diameter of 208 ± 21.3 nm. Future work will evaluate surface tension effects Pluronic of different PEO-PPO ratios to further optimize the NB formulation.

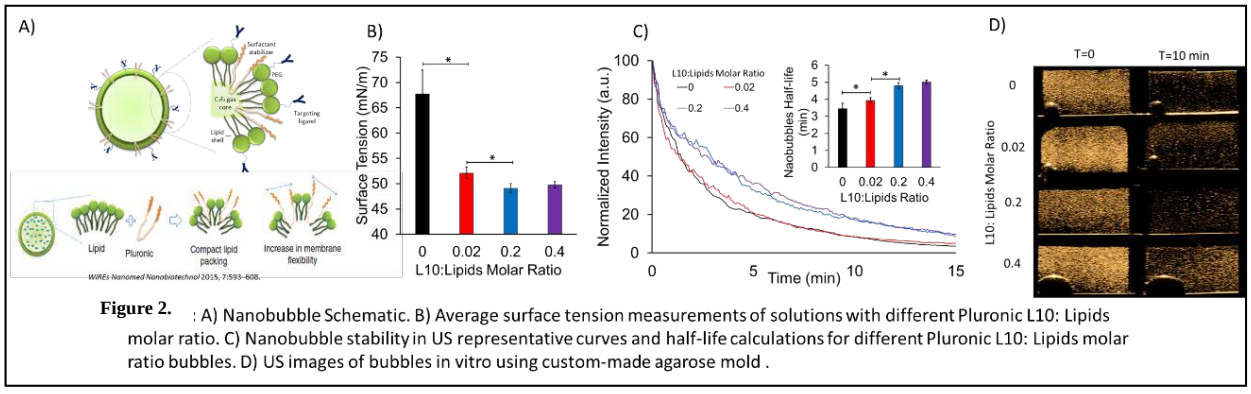


Figure 2. : A) Nanobubble Schematic. B) Average surface tension measurements of solutions with different Pluronic L10: Lipids molar ratio. C) Nanobubble stability in US representative curves and half-life calculations for different Pluronic L10: Lipids molar ratio bubbles. D) US images of bubbles in vitro using custom-made agarose mold .

1.3. On the Fate of Mesh-stabilized Lipid Nanobubbles after Destruction with Ultrasound

The dissipation of ultrasound signal from microbubble contrast agents has been linked to their fragmentation, jetting or sonic cracking, leading to a loss of gas. With strong interest in the use of bubbles in vivo, their ultimate fate is of great importance. It has been hypothesized that remnant shells shed into the surround aqueous medium, folding into liposomes or micelles. To investigate these effects, we have applied cryogenic transmission electron microscopy (cryo-EM) to image nanoscale lipid and polymer-stabilized perfluorocarbon gas bubbles before and after their destruction with high intensity US.

Methods: Polymer-stabilized lipid nanobubbles (NBs) were made by agitation of a lipid solution(DPPC:DPPE:DPPA), Pluronic L10, acrylamide monomers, crosslinker and irgacure 2959 in the presence of C_3F_8 gas, followed by crosslinking under U.V. light. Bubbles were imaged in an agarose mold in PBS using contrast harmonic imaging (Toshiba, 12 MHz, MI 0.1). Flash/replenish (20 cycles) was used to destroy bubbles. Particle size was determined by dynamic light scattering. For cryo-EM, NBs were applied to EM grids (R2/2, 400 mesh; EMS) glow-discharged for 30 sec at 15 mA and imaged on JEOL 2200FS transmission electron microscope with a total electron dose of <100 e^-/A^2.

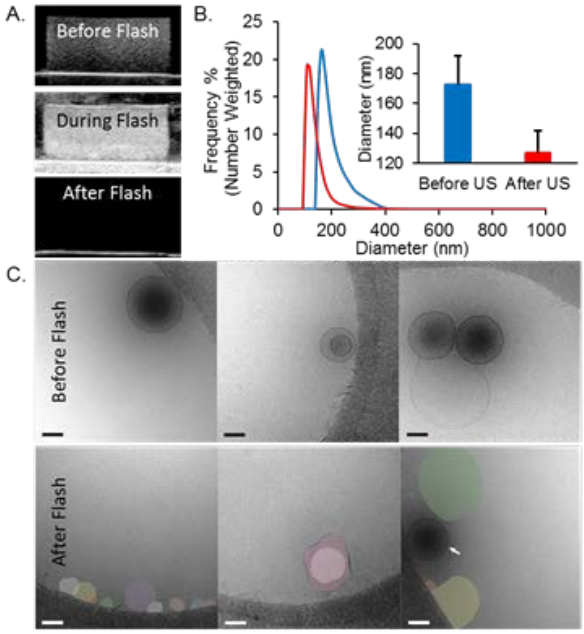


Figure 3. Change in nanobubble (A) US signal, (B) size and (C) shape (by cryo-EM) before and after destruction with high intensity US. Color added to cryoEM images for better visualization. Scale bars represent 100nm.

Results and Discussion: The application of the high intensity US was found to destroy all bubble contrast (**Fig 3a**). Mean NB diameter was significantly reduced from 172.7 ± 19.3 nm to 126.7 ± 15.0 nm suggesting a loss of gas from the particles (**Fig 3b**). Cryo-EM images of NBs demonstrate that particles have a monolayer shell with a dark center that is likely due to the higher density of the frozen C_3F_8 core relative to the surrounding water layer. Sonicated NBs appeared as amorphous and transparent lipid sheets, indicating a loss of gas (**Fig 3c**). While some multi-laminar vesicles were present in the NB solution prior to sonication, none could be visualized in the US disrupted solution. These results suggest that US-disrupted NBs do not reform as liposomes or micelles but rather flatten into round sheets following gas loss. This unexpected result may be due to the hydrophobic acrylamide core helping to maintain particle structure.

1.4. Ultrasound Signal from Sub-Micron Lipid-coated Bubbles

It remains unclear whether nanoscale lipid-stabilized bubbles can produce the observed enhanced acoustic backscatter at clinically relevant frequencies. Because NB polydispersity is high, micron sized bubbles are thought to contribute to experimental observations. Accordingly, this study examined echogenicity of lipid- and surfactant-stabilized perfluoropropane (C_3F_8) nanobubbles (NBs), rigorously controlling for the presence of microbubbles in the solution.

Methods**:** NBs were formulated by mechanical agitation of a PBS solution of lipids (DBPC:DPPE:DPPA), Pluronic L10, and glycerol in the presence of C_3F_8 gas. Microbubbles were separated from NBs based on their buoyancy by centrifugation. According to the Stokes equation (**Fig 4A**), when centrifuged at 50g for 5 min a bubble larger than 0.7 um should rise a distance of 0.5 cm. Care was taken to only collect samples below this distance. Particle size and buoyant mass were measured using Resonant Mass Measurement (RMM), Archimedes, Malvern Instruments). Isolated NBs were filtered using track-etched polycarbonate filters of 1000 nm and 400 nm using

a syringe pump at 0.125 mL/min and 0.5 mL/min and were imaged in an agarose mold in PBS using contrast harmonic imaging (Toshiba, 12 MHz, MI 0.1).

Results and Discussion: RMM results (**Fig. 4B**) show that mean NB size was 469 nm and all buoyant particles (negative mass) were below 1 µm. These large positive mass particles are likely large lipid aggregates and should not produce ultrasound signal. US images of isolated NBs (**Fig. 4C**) demonstrate considerable echogenicity. A reduction in signal after filtration was noted (**Fig 4C**), but NB activity was observed under all conditions. It is not clear whether the signal decrease is due to lower NB concentration or smaller bubble size. It is likely that filter pore blockage by lipid aggregates is responsible for some of the signal loss. Figure 4E shows microscopy and Nanosight particle size distribution following centrifugation. No particles larger than 700 nm were seen. While the mechanism of the strong NB activity at 12 MHz is not fully understood, a reduction of surface tension by the surfactant Pluronic, or buckling of the shell in response the US field, are likely contributors.

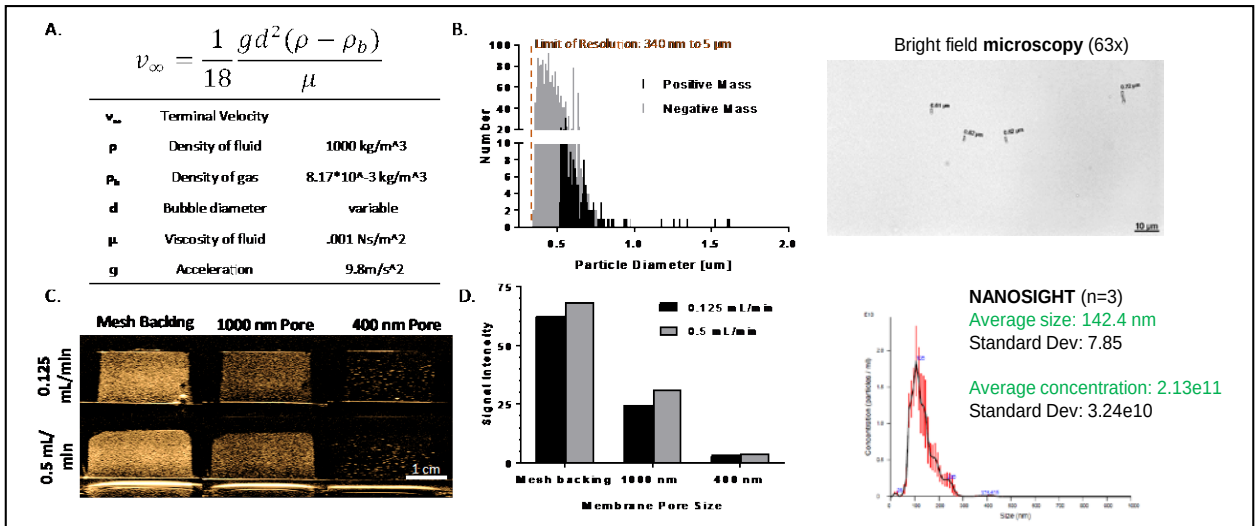


Figure 4. (A) Stokes law for the terminal velocity of a bubble. (B) Particle size distribution based on buoyancy determined by RMM with a lower limit of detection at 340 nm. US images (C) and corresponding grayscale intensity (D) of isolated NBs before and after being filtered through 1000 nm and 400 nm pores. (E) Microscopy and particle size analysis following centrifugation.

1.5. Characterization of PSMA-1-cys peptide synthesis and lipid conjugation via MALDI-TOF and Ellman's analysis

The lipids (DSPE-PEG, DSPE-PEG-MAL and L-cysteine were dissolved in 0.1M phosphate buffer. L-cysteine was added to the DSPE-PE and DSPE-PEG-MAL groups and the samples were evaluated for thiol content using Ellman's assay. Two different MAL:Thiol ratios were used (2:1 and 2:0.5). It is evident from **Fig. 5A**, that the reaction takes place rapidly, and no change is seen after 10 minutes. The level of thiol groups in the 2:0.5 ratio group decreased to control levels

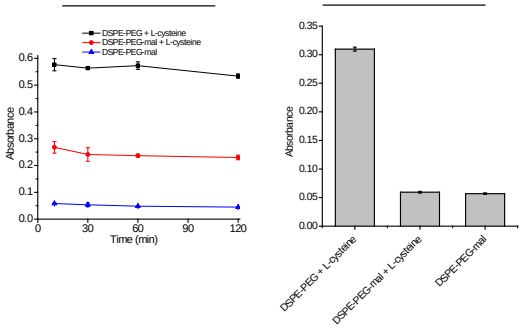
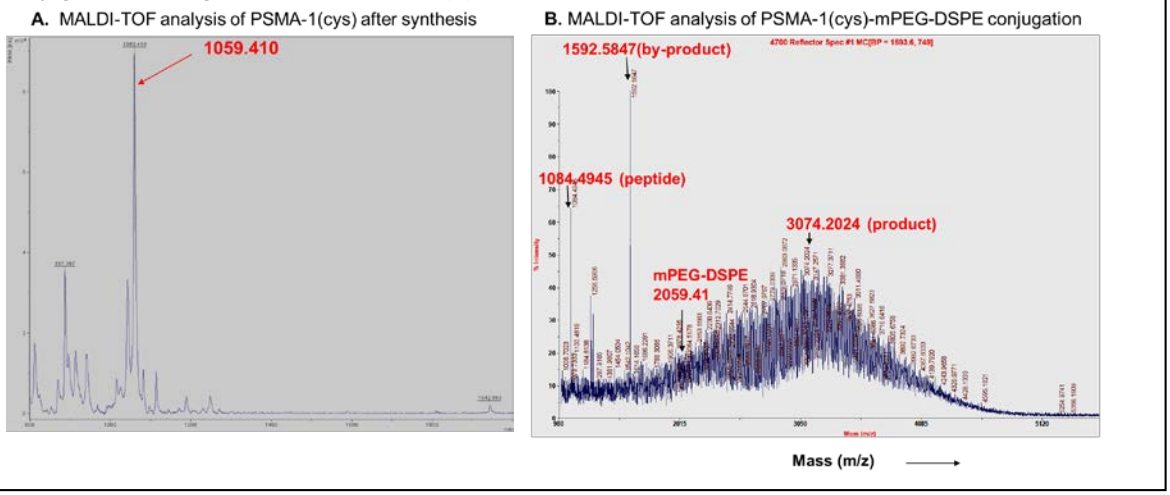


Figure 5. Confirming efficiency of lipid-mal-cys

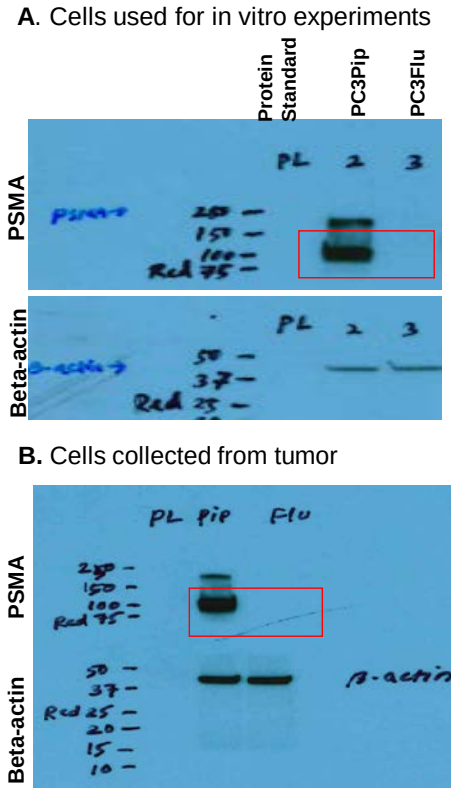
after 10 min (**Fig. 5B**), indicating near complete binding of cysteine to the DSPE-PEG-MAL group. Characterization of PSMA-1-cys peptide synthesis and lipid conjugation to PSMA-1-cys was confirmed by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis. The results are shown in **Fig 6A-B**.

Figure 6. MALID-TOF analysis confirming the successful synthesis of PSMA-1-cys ligand (**A**) and of the successful conjugation of the ligand to DSPE-PEG-Mal (**B**).



1.6. Confirmation of PSMA biomaker expression in cell lines via Western blotting

Figure 7. Confirmation of PSMA expression in cells (**A**) and tumors (**B**)



Methods: PC3pip (PSMA positive) and PC3Flu (PSMA negative) cells were grown to the logarithmic phase, rinsed with phosphate-buffered saline (PBS), placed on ice, and suspended in 200 µl of radioimmunoprecipitation assay (RIPA) protein lysis buffer. Next, all cells/tumor lysates were transferred to a 1.5-mL tube and centrifuged at 12000 rpm and 4°C for 15 min. The resulting supernatant was transferred to a new 1.5 mL centrifuge tube. A bicinchoninic acid (BCA) kit was then used to determine the protein concentration. Additionally, the samples were supplemented with 2X Laemmli loading buffer, mixed and boiled for 5 min to fully denature the proteins. Twenty micrograms of total protein was separated via SDS-PAGE and transferred to a nitrocellulose membrane via the semi-dry blotting method. Membranes were blocked with 5% milk in Tris Buffered Saline-Tween 20 (TBST) for 1 hour at room temperature. PSMA was detected with mAb J591 0.2 mg/mL for 1 hour followed by incubation with horseradish peroxidase-goat–anti-mouse IgG antibody (1:5,000 dilution) for 1 hour. After 3 TBST washes, blots were visualized by chemiluminescence. **Fig 7** confimrs expression of PSMA in both the PC3pip cell line as well as tumors grown from PC3pip cells. Lack of expression is seen in the PC3flu lines in both experiments.

Competition Binding Assay

Methods: Cells ($5X10^5$) were incubated with free PSMA-cys/ PSMA-1-NB and N-[N-[(S)-1,3-dicarboxypropyl]carbamoyl]-S-[3H]-methyl-L-cysteine (3H-ZJ24; GE Healthcare Life Sciences) in a total volume of 200 mL of 50 mmol/L Tris (pH 7.5) for 1 hour at 37 deg C. The mixture was centrifuged at 3,000 g for 5 minutes at 4 deg C to separate bound and free 3H-ZJ24. The supernatant was removed, and the cell pellet was washed 3 times with 500 mL of cold Tris buffer. Four milliliters of ECOLUME scintillation cocktail (MP Biomedicals) was added, and radioactivity was counted. Data were analyzed using GraphPad Prism 3.0.

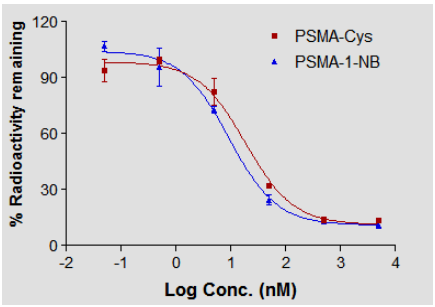
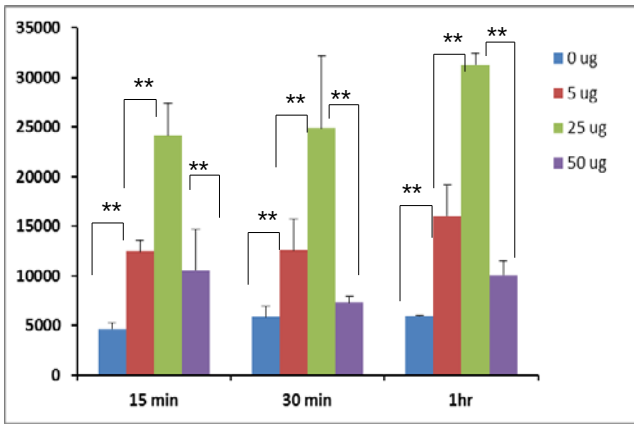


Figure 8. Competitive binding assay of PSMA-1 conjugated NBs where NB1 and NB2 constituted different methods of formulation of bubbles. IC50 values for PSMA-Cys and PSMA-1-NB were 18.6 nM and 9.2 nM

Results: Results show that the IC50 of PSMA-1- NB is lower (9.2 nM) than the ligand PSMA-1 (18.6 nM) (**Fig. 8**) in PSMA positive LNCaP cells.

1.7. In vitro binding studies (optimization of ligand density and cell microscopy)

Methods: Lipid conjugation of PSMA-Cys was performed through the -SH group of cysteine. PSMA-Cys was dissolved in anhydrous DMSO, to which 2.5-fold excess amount of Maleimide-PEG(2k)-DSPE was added. To formulate NBs, lipids DPPC, DPPE, DPPA and DSPE-PEG-PSMA-1 were dissolved in chloroform at a 4:1:1:1 ratio, dried and hydrated in PBS with Pluronic L10 solution. For optimization of binding, 40,000 cell/well were seeded 24 hr. before experiments. Bubbles with varying amounts of PSMA-cys ligand (0, 5, 25 and 50 ug (by weight)) were added and incubated for 15, 30 and 60 min. Rhodamine-DSPE was used to label to NBs. Cells were then washed with PBS and fluorescence per well analyzed with a plate reader.



*Statistical analysis is done by student's t-test where **P<0.01*

Figure 9. Binding optimization of PSMA-1-NBs (fluorescence from rhodamine-DSPE) in PC3pip cells. Data were obtained from fluorescence plate reader analysis.

For microscopy studies, 800,000 cells were seeded in 35 mm dish 24 hr before the experiment. Each dish contained one glass cover slip. On the day of experiment cells were washed by PBS for at least 2 times. Then the cells were incubated at 37 deg C either with unmodified or modified NBs for an hour. Cells were washed 2 times with PBS after incubation and fixed with 4% paraformaldehyde for 10 min. The cover slips were transferred to new dish and washed again for 3 times with PBS.

Finally cell nucleus was tagged with DAPI and slides were prepared with the cover slips. Image was taken with LEICA fluorescence microscope 25X.

Results: NBs were fluorescently tagged by adding rhodamine-DSPE into the lipid film. As evident in **Fig. 9**, nanobubbles containing 25 ug of PSMA-cys are better in targeting than other formulations. To determine cell binding, PSMA-expressing cells (PC3pip) and cells that do not express PSMA (PC3flu) were seeded ($1x10^6$ cells) onto 35mm dishes containing a cover slip 24 hrs prior to the experiment. Incubation of NBs with cells in culture for 60 min showed that targeted NBs accumulated significantly higher in PC3pip cells (**Fig. 10**). Data were collected using a fluorescent microscope and images processed to quantify fluorescence signal in cells. These preliminary *in vitro* data suggest that these PSMA-1 functionalized NBs indeed are able to bind cells expressing PSMA biomarker.

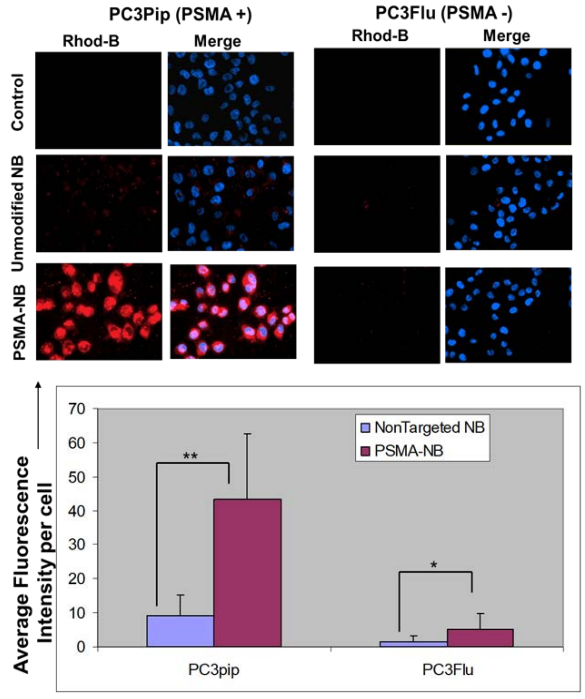


Figure 10. Binding of PSMA-1-NBs (fluorescence from rhodamine-DSPE) in PC3pip and PC3flu cells (A) and the respective quantification of microscopy data (B).

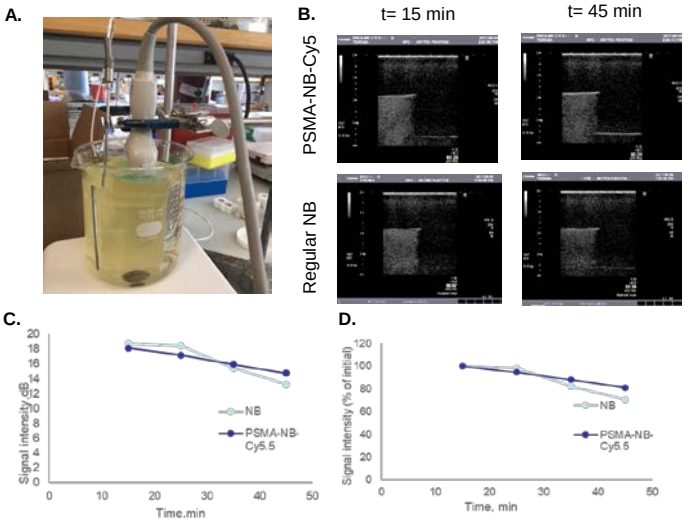


Figure 11. Acoustic characterization of NBs in phantom setup (A). Images were acquired with clinical ultrasound (B). Experiments compared the signal intensity (C) and signal decay (D) for PMSA and cy5.5 functionalized and non-functionalized nanobubbles. No differences were seen between groups.

1.8. Acoustic characterization of PSMA-1-NB

Following conjugation of the PSMA-1-cys ligand to DSPE-PEG-MAL lipid, we formulated nanobubbles using standard procedures. Initially, we did not stabilize these with polymers, because we wanted to explore the simpler bubble formulation strategy. Nanobubbles were then assessed via ultrasound imaging for their initial signal intensity as well as signal decay over time.

Methods: These experiments were conducted at physiological temperature, in a stirred system depicted in **Fig 11**. Bubbles were diluted into the PBS bath, and US

images were acquired at 1 frame every 10 seconds for the first 5 min, followed by periodic imaging for 30 minutes. A 12 MHz contrast harmonic imaging protocol was used, as described above. The decay and initial signal of functionalized nanobubbles also labeled with Cy5.5 fluorescent probe to that of standard, non-functionalized nanobubbles, to determine whether the surface decoration de-stabilized the bubbles or lead to a reduction in echogenicity.

Results: Both bubble types showed good stability over time and a 20% signal decay over 45 minutes. There was no difference seen with functionalized bubbles. This suggests that the addition of PSMA-1 ligand and the fluorescent label do not alter the signal intensity or the stability of the nanobubbles.

2. Overview of research accomplished pertaining to Task 2

The IACUC protocol has been approved, and we have begun to carry out initial in vivo experiments. Initial testing focused on establishing the appropriate tumor model geometry (location of the biomarker positive and negative tumors), and determining the PSMA-1 binding/uptake in both tumor types via optical imaging. In addition, we carried out ultrasound experiments comparing the kinetics of nanobubbles to that of commercially available microbubbles. These experiments serve to determine whether non-functionalized nanobubbles behave differently in the PC3 tumors then larger bubbles.

2.1. Confirming PSMA expression in vivo

Tumors were Mouse tumor xenograft models: All animal procedures were performed according to Institutional Animal Care and Use Committee (IACUA)-approved protocols. For flank tumor xenografts, 6- to 8-week-old athymic nude mice were implanted subcutaneously with 1×10^6 of PSMA-negative PC3flu and PSMA-positive PC3pip cells in 75 µL Matrigel on the right leg. Animals were observed every other day until tumors reached at about 5-6 mm in diameter. After 2 weeks, animals were ready for experiment.

In vivo imaging studies: Imaging was performed with the aid of the Maestro Imaging System (Perkin-Elmer) with each mouse receiving 1 nmol of NIR probe in PBS through tail vein injection. Imaging was performed at different time points using the appropriate filter set (deep red filter set for PSMA-1–IR800). During imaging, the temperature of imaging bed was adjusted to 37°C. Mice received inhalation of isofluorane through a nose cone attached to the imaging bed. Mice were imaged over 24 hr post injection.

Results are shown in **Fig 12.** The PSMA-1-IR800 can be seen accumulating in the Pc3pip tumors but not in the Pc3flu tumors. The peak accumulation was at 2.5 hours following injection. This experiment confirmed that the tumors express the biomarker and can be imaged with optical imaging. Next steps include repeating these experiments with fluorescent PSMA-1-NBs. These studies are currently in the planning stages and will commence later this year.

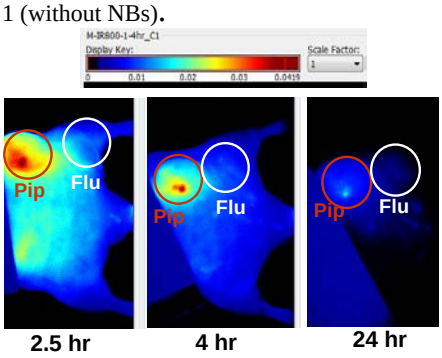


Figure 12. Optical imaging of PC3pip and PC3flu tumors grown adjacent to each other in mouse flank. Images were acquired following IV injection of fluorescent PSMA-1 (without NBs).

2.2. Nanobubbles Enhance Ultrasound Imaging of Prostate Tumors in Mice

The most widely used ultrasound contrast agents are lipid or protein-stabilized perfluorocarbon (PFC) gas microbubbles (MB) typically exceeding 2µm in diameter. These bubbles usually show rapid transient tumor enhancement, as they are confined to vasculature. To achieve longer lasting enhancement and improved delineation of tumors, we developed sub-micron lipid and surfactant-stabilized PFC nanobubbles (NB). Here we compared tumor kinetics of the NBs compared to commercially available MBs.

Methods: C_3F_8 NBs were formulated by dissolving a cocktail of lipids including DBPC, DSPE-PEG in PBS followed by gas exchange and activation via mechanical agitation. NBs were purified by centrifugation, and size was measured by dynamic light scattering (DLS). Tumors were inoculated in the flank of three male nude mice by injection of PC3 prostate cancer cells in Matrigel®, and grown to 5-8 mm (**Fig. 13A**). Contrast-enhanced US images were acquired with Vevo 3100 (Visualsonics Fujifilm) at 1fps, 18MHz, and 4% power following tail vein injections of 100 ul of either MicroMarker (Visualsonics) or NBs. Maximum intensity projection (MIP) and time-intensity curves (TIC) were obtained in the same mouse for both contrast agents.

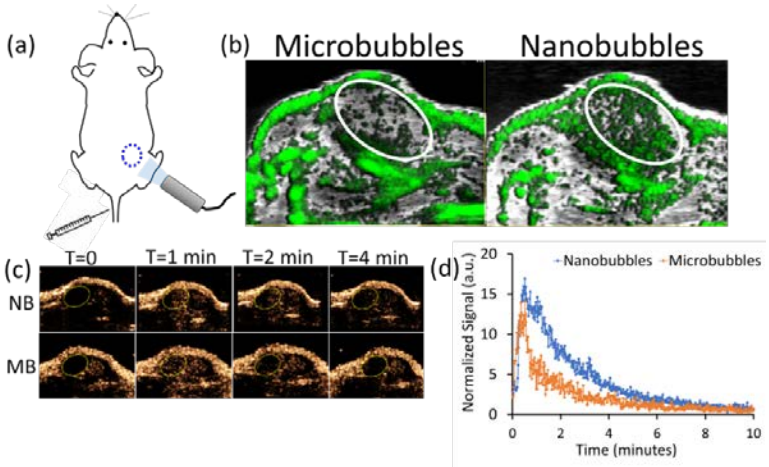


Figure 13: (a) Schematic of animal tumor model and US scan orientation. (b) MIP comparison in PC3 flank tumor 15s after contrast injection. (c) Representative tumor images of NBs and MBs from the same mouse. (d) Mean TIC curves for NBs and MBs.

Results/Discussion: NBs have a diameter of 240±95 nm, (compared to 2-3µm for MicroMarker). MIP images (**Fig. 13B**) show that NB provided more signal throughout the tumor cross section compared to MBs at t=15s. Representative contrast images are shown in **Fig. 13C** and the mean TIC for all replicates is shown in **Fig. 13D.** NBs had a half-life of 2.1min compared to 1min for microbubbles, and at t=2 min showed a signal intensity nearly 3 times higher than MBs. Higher tumor signal and slower wash out suggests that smaller NBs were able to penetrate out of the leaky tumor vasculature and further into the tumor interstitium. Such NBs may eventually provide a more effective contrast agent compared to MBs and could enhance US guided biopsies.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

We hired two postdoctoral fellows, Drs. Jacob Liliy and Afsana Akhter as part of this project. This was the first postdoctoral training experience for both individuals, and they have obtained advanced professional skills training via various interactions with mentors, attended seminars, supervised graduate and supervised undergraduate students.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

We are currently mentoring two high school students from local Cleveland schools in the laboratory. They have exposure to all of the projects in our laboratory including this one.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

As Tasks 1-2 summarize, in the next reporting period, we plan to continue carrying out NB formulation optimization to further reduce the size, increase stability of ultrasound signal and maximize cell binding and uptake in vitro. We also plan to carry out many more of the in vivo tests in mouse models to examine the biodistribution of fluorescent NBs conjugated to PSMA-1 compared to un-targeted NBs in PSMA+ and PSMA- tumors with optical imaging and ultrasound.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Currently, we are only 30% into the project, and we have not yet had the opportunity to determine the broader impact of this research on our discipline or other disciplines. In general, we are intending to broaden the scope of ultrasound imaging applications that can have true patient impact. This includes developing additional molecular imaging applications, and better nanobubble formulations. We are also working to better understand the fundamental biophysical and acoustical reasons behind the unique NB behavior. This will take some additional time.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We had initial issues with the (PC3pip) cells which express and do not express (PC3flu) PSMA biomarker being cross-contaminated. Steps were immediately taken to resolve the issues, and, as seen in the biomarker expression results, we have now resolved the contamination issues. We do not expect additional problems to arise, but will test PSMA expression in our cell lines periodically to confirm.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Hernandez C, Gulati S, Stewart P, Exner AA. Cryo-EM Visualization of Lipid and Polymer-Stabilized Perfluorocarbon Gas Nanobubbles. Scientific Reports. In preparation.

  Nieves L, Exner AA. Effect of the Surfactant Pluronic on the Stability of Lipid-Stabilized Perfluorocarbon Nanobubbles. IEEE International Ultrasonics Symposium 2017 Proceedings. *In preparation.*

  Hernandez C Exner AA. Ultrasound Signal from Sub-Micron Lipid-coated Bubbles. IEEE International Ultrasonics Symposium 2017 Proceedings. *In preparation.*

  Hernandez C, Gulati S, Stewart P, Exner AA. On the Fate of Mesh-stabilized Lipid Nanobubbles after Destruction with Ultrasound. IEEE International Ultrasonics Symposium 2017 Proceedings. *In preparation.*

  Lilly J, Xia H, Ankher A, Ramamamurthy G, Basilion J, Exner AA. Nanobubble Contrast Agents Enhance Ultrasound Imaging of Prostate Tumors in Mice. IEEE International Ultrasonics Symposium 2017 Proceedings. *In preparation.*

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

1. Afsana Akhter, Jacob Lilly, Christopher Hernandez, Gopalkrishnan Ramamurthy, Hansheng Xia, Xinning Wang, Agata A. Exner, James P. Basilion. In vitro assessment of a novel PSMA-targeted nanobubble for prostate cancer ultrasound imaging. World Molecular Imaging Congress. 2017. *Under review.*
2. C Hernandez, L Nieves, J Lilly, H Xia, A Ankher, X Wang, G Ramamurthy, R Advincula, J Basilion, MC Kolios, AA Exner. Toward successful ultrasound molecular imaging of cancer with nanobubble contrast agents. Contrast Media Research 2017. *Under review.*
3. Gabriella Fioravanti, Christopher Hernandez, Agata A. Exner. Lipid Acyl Chain Length Improves Stability of Nano-sized Ultrasound Contrast Agents In Vitro. Biomedical Engineering Society 2017 meeting. Under review.
4. Nieves L, Exner AA. Effect of the Surfactant Pluronic on the Stability of Lipid-Stabilized Perfluorocarbon Nanobubbles. IEEE International Ultrasonics Symposium 2017 Annual meeting. *Poster presentation.*
5. Hernandez C Exner AA. Ultrasound Signal from Sub-Micron Lipid-coated Bubbles. IEEE International Ultrasonics Symposium 2017 Annual meeting. *Poster presentation.*
6. Hernandez C, Gulati S, Stewart P, Exner AA. On the Fate of Mesh-stabilized Lipid Nanobubbles after Destruction with Ultrasound. IEEE International Ultrasonics Symposium 2017 Annual meeting. *Poster presentation.*
7. Lilly J, Xia H, Ankher A, Ramamamurthy G, Basilion J, Exner AA. Nanobubble Contrast Agents Enhance Ultrasound Imaging of Prostate Tumors in Mice. IEEE International Ultrasonics Symposium 2017 Annual meeting. *Oral presenation.*
8. Nieves L, Hernandez C, Exner AA. Structure-Function Relationship between Lipid Shell Components and Surface Tension of Nanobubble Ultrasound Contrast Agents; 2016 Annual Biomedical Research Conference for Minority Students (ABRCMS) Tampa, FL; *Poster Presentation*
9. Nieves L, Hernandez C, Exner AA. Structure-Function Relationship between Lipid Shell Components and Surface Tension of Nanobubble Ultrasound Contrast Agents; SFB Midwest 2016, *Oral presentation;* *Honorable Mention Award*
10. Fioravanti G, Hernandez C, Exner AA, Lipid Acyl Chain Length Improves Nanosized Contrast Agents Stability In Vitro; SFB Midwest 2016, *Oral presentation*
11. Hernandez C, Wang X, Basilion J, Exner AA. Early Detection of Prostate Cancer with New Nanoparticle-Based Ultrasound Contrast Agents Targeted to PSMA. 2016 World Molecular Imaging Conference. *Poster presentation.*

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Invention #: 2017-3125 Title: PSMA Targeted Nanobubbles for Diagnostic and Therapeutic Applications Patent filing: Provisional patent, 62/381,144 Filing date: August 30, 2016 Funding reported: DoD W81XWH-16-1-0371 Summary: This application relates to diagnostic and therapeutic compositions, and more particularly to targeted nanobubbles for diagnostic, therapeutic, and theranostic applications. Abstract: A PSMA targeted nanobubble includes a membrane that defines at least one internal void, which includes at least one gas, the membrane including at least one lipid, at least one nonionic triblock copolymer that is effective to control the size of the nanobubble without compromising in vitro and in vivo echogenicity of the nanobubble, and an interpenetrating cross-linking biodegradable polymer; and at least one PSMA ligand coupled or linked to the membrane.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

<u>Example:</u>

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Agata Exner PhD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	AEXNER (NIH Commons)
Nearest person month worked:	2.4
Contribution to Project:	Oversees project operations
Funding Support:	Current grant and NIH R01EB016960
Name:	James Basilion PhD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	JBASILION (NIH Commons)
Nearest person month worked:	1.4
Contribution to Project:	Oversees project operations
Funding Support:	Current grant and NIH
Name:	Jacob Lilly PhD
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	11
Contribution to Project:	Performed tasks pertinent to nanobubble formulation optimization and functionalization
Funding Support:	Current grant
Name:	Afsana Akhter PhD
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	11
Contribution to Project:	Performed tasks pertinent to PSMA ligand synthesis, conjugation, expression in cells, and in vivo models.
Funding Support:	Current grant
Name:	Xinning Wang
Project Role:	co-investigator
Researcher Identifier (NIH commons or ORCID ID):	Xining.wang (NIH Commons)
Nearest person month worked:	6 months
Contribution to Project:	synthesizing and purification of PSMA-1-Cys ligand; teaching the new postdoctoral how to make the peptide; Establish HPLC method to follow the reaction of PSMA-cys with DSPE maleimide; MALDI confirmation of the

	conjugation of PSMA-cys with the lipid; performing competition binding experiments for the ligands and nanobubbles, providing PSMA-1-IR800 for in vivo imaging experiments.
Funding Support:	this funding and RO1EB020353, RO1, NIHNIBIB

Name:	Lenitza Nieves
Project Role:	Lab technician
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	7
Contribution to Project:	Carried out fundamental biophysical experiments to determine the bubble shell properties.
Funding Support:	CWRU NIH PREP program R25GM075207
Name:	Chris Hernandez
Project Role:	Graduate student
Researcher Identifier (e.g. ORCID ID):	CHRISTOPHER.HERNANDEZ
Nearest person month worked:	1.5
Contribution to Project:	Basic acoustic characterization and optimization of the nanobubbles
Funding Support:	F31 CA200373-01
Name:	Hansheng Xia
Project Role:	Visiting research fellow
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	5
Contribution to Project:	Carried out ultrasound scans on agents in vitro and in vivo. Assisted with experiments
Funding Support:	Self-funded from China.

Name:	Gabby Fioravanti
Project Role:	Undergraduate researcher
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	4
Contribution to Project:	Carried out experiments to determined shell property effects on bubble stability
Funding Support:	Pilot grant from Case Comprehensive Cancer Center P30CA043703 R01EB016960

H**as there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Exner: MPI on Mary Kay Foundation (MPI with Brady-Kalnay, DiFeo)
PTPmu as a targeting agent for ultrasound-mediated deployment and treatment of ovarian cancer. 7/1/2017-6/30/2019

Basilion: Nothing to report.

Ponsky: Co-investigator on 1R01CA208236-01A1; Project Start Date: 2-MAR-2017
Contact PI / Project Leader: GULANI, VIKAS
Title: MR FINGERPRINTING AND COMPUTERIZED DECISION SUPPORT FOR PROSTATE CANCER

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc.,*

available to project staff);

- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

I have included my CV.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.

Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Basilion, James Peter

eRA COMMONS USER NAME (agency login): JBASILION

POSITION TITLE: Professor of Radiology and Biomedical Engineering

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
University of Pennsylvania, Philadelphia, PA	BFA	06/1984	Biochemistry
University of Texas Graduate School of Biomedical Sciences, Houston, TX	PHD	06/1990	Molecular Pharmacology
National Institutes of Health, NICHD, Bethesda, MD	Postdoctoral Fellow	08/1994	Staff Fellow-Post doctoral training studying post-transcriptional gene regulation
National Institutes of Health-NICHD, Bethesda, MD	Postdoctoral Fellow	02/1996	IRTA-Postdoctoral training in post-transcriptional gene regulation

A. PERSONAL STATEMENT

I am a molecular biologist/pharmacologist who had worked at CMIR/MGH with Dr. Weissleder on the development of imaging agents for several years and have gotten insights into the issues surrounding probe design and pharmacology, particularly with optical imaging agents. Eleven years ago I moved to Case Western Reserve University to become faculty at both the Case Center for Imaging Research (Department of Radiology, Case Medical School) and at the Department of Biomedical Engineering (Case School of Engineering). I am Vice-Chairman of Basic Science Research for the Department of Radiology and co-direct the Cancer Imaging Program for the Case Comprehensive Cancer Center, and direct both the NFCR Center for Molecular Imaging at Case and the Case Center for Imaging Research. My academic research career has been devoted to the study and understanding of different diseases for the purposes of detecting them earlier and effecting surgical or other cures. Our lab studies imaging of parasitic disease, of various and numerous different human cancers, of different inflammatory responses, and we have developed technologies to identify and image molecular profiles of said diseases. The common thread to these studies has always been molecular imaging and development of novel imaging probes and paradigms to image these diseases non-invasively and in real time. For our research we utilize most imaging modalities including, MRI, PET, and optical imaging.

For the past few years I have developed significant expertise within our laboratory in the development and implementation of prostate specific membrane antigen (PSMA)-targeted imaging agents making my lab qualified to help perform and manage these studies. My research career has been hybrid consisting of experience in the biotech industry in addition to my academic position, which started in the late 1990s. My experience in industry has influenced my perspectives and my laboratory conducts approximately 70% translational research. My industrial experience additionally has given me managerial skills very important to participate in this multi-component, multi-partner research project.

B. POSITIONS AND HONORS

Positions and Employment

1989 - 1989 Consultant, Allergan Pharmaceutical, Irvine, CA
1990 - 1997 Consultant, Janux Group Inc., Greenbelt , MD
1996 - 1999 Senior Scientist, Variagenics, Inc., Cambridge, MA

1996 - 1999	Visiting Research Scientists, Harvard Medical School, Charlestown, MA
1999 - 1999	Consultant, Variagenics Inc., Cambridge, OH
1999 - 2005	Staff Scientist (Molecular Biology), CMIR Massachusetts General Hospital, Charlestown, MA
1999 - 2005	Assistant Professor of Radiology, Harvard Medical School, Charlestown, MA
2000 - 2005	Co-Director, National Foundation for Cancer Research, Center for Molecular Analysis and Imaging, Massachusetts General Hospital/Harvard Medical School, Charlestown, MA
2001 - 2001	Consultant and member of Scientific Advisory Board, UBS Warburg Healthcare Group, New York, NY
2002 - 2002	Consultant , Novartis Pharma AG BU Oncology
2005 -	Director, National Foundation for Cancer Research Center for Molecular Imaging, Case Western Reserve University, Cleveland, OH
2005 - 2013	Associate Professor of Radiology, Case Western Reserve University, School of Medicine, Cleveland, OH
2005 - 2013	Associate Professor of Biomedical Engineering, Case Western Reserve University, Case School of Engineering, Cleveland, OH
2007 - 2007	Consultant, Member Oncology Imaging Scientific Advisory Board, Eli Lilly & Company, IN
2008 -	Co-founder and Board of Directors, Akrotome Imaging Inc., Cleveland , OH
2008 -	Consultant , Akrotome Imaging Inc., Cleveland , OH
2009 -	Co-Director, Cancer Imaging Program, Case Comprehensive Cancer Center, Cleveland, OH
2013 -	Tenured Professor of Radiology, Case Western Reserve University, School of Medicine, Cleveland , OH
2013 -	Tenured Professor of Biomedical Engineering, Case Western Reserve University, Case School of Engineering, Cleveland, OH
2013 -	Professor of Pathology (adjunct), Case Western Reserve University, School of Medicine, Cleveland, OH
2013 -	Professor Cancer Center, Case Comprehensive Cancer Center, Cleveland, OH
2013 -	Co-Director (Interim), Case Center for Imaging Research, Case Western Reserve University, School of Medicine, Dept. of Radiology, Cleveland, OH
2015 –	Scientific Advisory Board, Lightpoint Medical Ltd.
2016 -	Scientific Advisory Board, Vergent Biosciences Inc.

Other Experience and Professional Memberships

2000 - 2011	Founding Member, Society For Molecular Imaging (SMI)
2002 -	Member, American Association for Cancer Research
2005 -	Founding Member, American Academy of Nanomedicine
2006 -	Member, Institute of EIInstitute of Electrical and Electronics Engineers (IEEE)
2006 -	Member, American Association for the Advancement of Science (AAAS)
2009 - 2009	Charter Member, American Society for Nanomedicine (ASNM)
2010 -	Member, Biomedical Engineering Society (BMES)
2011 -	Founding member (Treasurer 2011-2013), World Molecular Imaging Society (WMIS) (merger of AMI and SMI)
2012 -	Co-Director Optical Surgical Navigation Interest Group at WMIS
2016 -	President Elect World Molecular Imaging Society

Honors

1989	Award for best oral presentation, 13th Annual Meeting of TX Pharmacologists
2013	Distinguished Investigator Award, Academy of Radiology Research
2014	Elected Fellow, American Institute for Medical and Biomedical Engineering (AIMBE)

C. Contribution to Science

1. **Research in MR Imaging Agents:** This began with the development of a MR contrast agent to image gene therapy and provide non-invasive monitoring of cell surface receptor levels. My work "In Vivo magnetic resonance imaging of transgene expression". Weissleder, R.,……Basilion, J.P., Nature Medicine (2000), was among the first to demonstrate that an engineered cellular protein could be placed in a cell and MRI could be used to detect that marker in tumors in vivo. It provided the framework to allow the field to think about MRI as a molecular imaging tool and the framework to begin to define the parameters that needed to be optimized to amplify the signal for detection by MRI. This work has been sited over 800 times. Developing MRI as a molecular imaging tool has continued within my laboratory.
   a. Weissleder R, Moore A, Mahmood U, Bhorade R, Benveniste H, Chiocca, E.A., **Basilion, J.P.** In vivo magnetic resonance imaging of transgene expression. Nat Med. 2000 Mar;6(3):351-5. PubMed PMID: 10700241.
   b. Högemann D, Josephson L, Weissleder R, **Basilion JP**. Improvement of MRI probes to allow efficient detection of gene expression. Bioconjug Chem. 2000 Nov-Dec;11(6):941-6. PubMed PMID: 11087345.
   c. Moore A, Josephson L, Bhorade RM, **Basilion JP**, Weissleder R. Human transferrin receptor gene as a marker gene for MR imaging. Radiology. 2001 Oct;221(1):244-50. PubMed PMID: 11568347.
   d. Perera VS, Yang LD, Hao J, Chen G, Erokwu BO, Flask, CA, Zavalij, PY, **Basilion, JP**, Huang, SD. Biocompatible nanoparticles of KGd(H [illegible] Extremely high T1-weighted relaxivity owing to two water molecules directly bound to the Gd(III) center. Langmuir. 2014 Oct 14;30(40):12018-26. PubMed PMID: 25238130; PubMed Central PMCID: PMC4196746.
2. **Theranostic Nanotechnology:** My laboratory has also used nanoparticles to develop theragnostic agents, moleculaes which can both detect and treat brain tumors. These particles can be used to inform surgical resection of brain tumors as well as provide therapy after surgery is complete. In collaboration with Clemens Burda we have utilized gold nanoparticles to deliver a hydrophobic photodynamic therapy (PDT) agent, PC4, across the blood-brain-tumor-barrier to orthotopically implanted brain tumors (Yu….Basilion, Small, (2011). PC4 is fluorescent and therefore can be used to visualize the brain tumor for surgical guidance. Following surgery, the surgical field can be irradiated with red light activating the drug to kill cancer cells. We are also working with Dr. Karathanasis to develop nano chains capable of delivering therapeutic agents to brain tumors. Finally we have developed targeted small molecules that include Pc4 enabling another theranostic approach for tumor resections.
   a. Labhasetwar V, Zborowski M, Abramson AR, **Basilion JP**. Nanoparticles for imaging, diagnosis, and therapeutics. Mol Pharm. 2009 Sep-Oct;6(5):1261-2. PubMed PMID: 19799461.
   b. Cheng Y, Meyers JD, Broome AM, Kenney ME, **Basilion JP**, et al. Deep penetration of a PDT drug into tumors by noncovalent drug-gold nanoparticle conjugates. J Am Chem Soc. 2011 Mar 2;133(8):2583-91. PubMed PMID: 21294543; PubMed Central PMCID: PMC3056176.
   c. Cheng Y, Meyers JD, Agnes RS, Doane TL, Kenney ME, Broome, A.-M., Burda, C. and **Basilion, J. P.** Addressing brain tumors with targeted gold nanoparticles: a new gold standard for hydrophobic drug delivery?. Small. 2011 Aug 22;7(16):2301-6. PubMed PMID: 21630446; PubMed Central PMCID: PMC3837553.
   d. Meyers, J, Cheng, Y, Broome, AM, Agnes, RS, Schluchter, MD, Margevicius, S, Wang, X, Kenney, ME, Burda, C, **Basilion, JP**. Peptide-Targeted Gold Nanoparticles for Photodynamic Therapy of Brain Cancer (2014) Particle & Particle Systems Characterization, Apr;32(4):448-457. PMCID: PMC4437573
   e. P.M. Peiris, A. Abramowski, J. Mcginnity, E. Doolittle, R. Toy, R. Gopalakrishnan, S. Shah, L. Bauer, K.B. Ghaghada, C. Hoimes, S. Brady-Kalnay, **J.P. Basilion,** M.A. Griswold, E. Karathanasis, Treatment of invasive brain tumors using a chain-like nanoparticle, *Cancer Research* 2015 Apr 1;75(7):1356-65. doi: 10.1158/0008-5472.CAN-14-1540. Epub 2015 Jan 27. PMID: 25627979
   f. Wang X, Tsui B, Ramamurthy G, Zhang P, Meyers J, Kenney ME, Kiechle J, Ponsky L, **Basilion** JP. Theranostic Agents for Photodynamic Therapy of Prostate Cancer by Targeting Prostate-Specific Membrane Antigen. Mol Cancer Ther. 2016 Aug;15(8):1834-44. doi: 10.1158/1535-7163.MCT-15-0722. Epub 2016 Jun 13. PMID: 27297866

3. **Intra-operative MI:** A major emphasis of my research program at Case is the development of technologies and tools for use in intra-operative imaging for real-time guidance during tumor resections. This work has created the development of targeted imaging agents for identification of tumors after IV injection. Additionally we have developed the novel approach of developing topically administered imaging agents to define tumor margins. It is paradigm shifting for how probes are applied to targets and defines a shorter clinical path to market for imaging agents and lowers potential side effects (lower doses are used and systemic distribution is negligible). This work has led to over $4.6M in funding to CWRU and the spin off of Akrotome Imaging Inc. to commercially develop topically administered agents for clinical use in both breast and skin cancer margin identification.
   a. Cutter JL, Cohen NT, Wang J, Sloan AE, Cohen AR, Panneerelvam, A., Schluchter, M., Blum, G., Bogyo, M., and **Basilion, J.P.** Topical application of activity-based probes for visualization of brain tumor tissue. PLoS One. 2012;7(3):e33060. PubMed PMID: 22427947; PubMed Central PMCID: PMC3302795.
   b. Agnes RS, Broome AM, Wang J, Verma A, Lavik K, and **Basilion, J.P.** An optical probe for noninvasive molecular imaging of orthotopic brain tumors overexpressing epidermal growth factor receptor. Mol Cancer Ther. 2012 Oct;11(10):2202-11. PubMed PMID: 22807580; PubMed Central PMCID: PMC3829608.
   c. Walker E, Gopalakrishnan R, Bogyo M, **Basilion J.P.** Microscopic detection of quenched activity-based optical imaging probes using an antibody detection system: localizing protease activity. Mol Imaging Biol. 2014 Oct;16(5):608-18. PubMed PMID: 24705781; PubMed Central PMCID: PMC4240007.
   d. Wang X, Huang SS, Heston WD, Guo H, Wang BC, **Basilion,JP.** Development of targeted near-infrared imaging agents for prostate cancer. Mol Cancer Ther. 2014 Nov;13(11):2595-606. PubMed PMID: 25239933.
   e. Walker, E., Mann, M., Honda, K., Vidimos, A., Schluchter, M., Straight, B., Bogyo, M., Popkin, D., and Basilion, J.P., Rapid Visualization of Non-Melanoma Skin Cancer. J.Am. Acad. Dermatology. 2016. *In press.*
4. **Imaging Complex Molecular Signatures:** Another major interest of my laboratory is to develop molecular imaging to meet future challenges. The state-of-the-art for molecular medicine has now has demonstrated that coupling changes in protein expression and protein modifications can lead to the characterization of disease-specific biological networks (molecular signatures) for diagnosis, drug selection, and prognosis. Our work in this area is laying the groundwork for eventual non-invasive imaging of diagnostic molecular signatures and thus will provide a global survey of the entire tumor rather than a subset of cancer cells obtained with biopsy.
   a. Högemann-Savellano D, Bos E, Blondet C, Sato F, Abe T, Josephson, L., Weissleder, R., Gaudet, J., Sgroi, D., Peters, P.J., and **Basilion, J.P.** The transferrin receptor: a potential molecular imaging marker for human cancer. Neoplasia. 2003 Nov-Dec;5(6):495-506. PubMed PMID: 14965443; PubMed Central PMCID: PMC1502574.
   b. Broome AM, Bhavsar N, Ramamurthy G, Newton G, **Basilion JP**. Expanding the utility of beta-galactosidase complementation: piece by piece. Mol Pharm. 2010 Feb 1;7(1):60-74. PubMed PMID: 19899815; PubMed Central PMCID: PMC2835542.
   c. Burden-Gulley SM, Qutaish MQ, Sullivant KE, Tan M, Craig SE, **Basilion J.P.**, Lu Z.R., Wilson D.L., Brady-Kalnay S.M. Single cell molecular recognition of migrating and invading tumor cells using a targeted fluorescent probe to receptor PTPmu. Int J Cancer. 2013 Apr 1;132(7):1624-32. PubMed PMID: 22987116; PubMed Central PMCID: PMC3558593.
   d. Broome AM, Ramamurthy G, Lavik K, Liggett A, Kinstlinger I, **Basilion, JP**. Optical Imaging of Targeted β-Galactosidase in Brain Tumors to Detect EGFR Levels. Bioconjug Chem. 2015 Mar 30;PubMed PMID: 25775241.

D. RESEARCH SUPPORT

2P30 CA043703-24 (Gerson, PI) NIH/NCI	08/01/97-03/31/18 $3,088,653	0.84 calendar

Case Comprehensive Cancer Center Support Grant
Goal: The objectives of the Center are: 1) to improve the prevention, diagnosis, and therapy of cancer through research; 2) to stimulate and support innovative, coordinated, interdisciplinary research on cancer diagnosis, treatment, and control; 3) to develop clinical applications of research discoveries and to make these applications available as quickly as possible; and 4) to develop cancer prevention and control activities to contribute to the reduction of cancer morbidity and mortality in Northeast Ohio and the surrounding region and nation. Role: Co-Leader, Cancer Imaging Program

1R01EB016960 (Exner, PI) 05/01/13-04/30/18 0.30 calendar
NIH/NIBIB $314,043
Pressure-Driven Local Drug Delivery System for Treatment of Liver Cancer
Goal: This proposal aims to improve the design of local injectable drug delivery systems for minimally invasive, image-guided cancer chemotherapy using a dual approach. Role: Co-Investigator

National Foundation for Cancer Research 01/01/2015-12/31/2016 1.8 calendar
NFCR Center for Molecular Imaging at Case $86,956 (no salary)
Effective Imaging of prostate cancer for staging and therapy
Goal: TO develop novel methodology for prostate cancer imaging and ablation using radio isotopes.
Role: PI

R01CA179956 (Brady-Kalnay) 09/01/2013-08/31/2016 0.27 calendar
NIH $377,593
(PQC5) Detecting small clusters of tumor cells with PT
Goal: To develop a magnetic resonance imaging (MRI) agent with great amplification potential and to use magnetic resonance (MR) acquisition and image processing techniques to provide contrast sufficient to detect even very small tumors. Role: Co-Investigator

U01 (Karathanasis, PI) 07/01/2015-06/30/2020 0.2 calendar
NIH/NCI $242,611
Treatment of gioblastoma using chain-like nanoparticles.
Goal: To develop a platform approach for delivery of effect drugs across the BBB to brain tumors, eradicating both GBM and tumor initiating cells. Role: Co-I

R01 SBIR/STTR (Akrotome) 09/01/2015-08/31/2017 1.2 calendar NIH
$378,550
Intraoperative assessment of non-melanoma skin cancer margins using NIRF probes
Goal: To develop and optimize a complete solution for assessment of tumor margins in skin cancer and perfrom a small clinical trial to validate the approach.
Role: Subcontract PI

R01EB020353 07/01/2016-06/30/2020 4.0 calendar
NIH/NIBIB $437,532
Theranostic gold nanoparticles for imaged-guided radical prostatectomy and PDT ablation
Goal: To develop nanoparticle delivery of theranostic agents for image-guided surgery and ablation of unresected prostate cancer,
Role: PI

DOD-IDEA Development Award 09/29/2016-09/28/2019 1.4 calendar DOD
$250,000
Early Detection of Prostate Cancer with New Nanoparticlebased Ultrasound Contrast Agents Targeted to PSMA
Goal: To develop targeted nanometer-scale bubble contrast agents for quantitative ultrasound imaging of prostate cancer eventually impacting the clinical management of the disease.
Role: Partnering PI